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13. ABSTRACT (Maximum 200 Words)

An increasing percentage of elderly women, particularly in industrialized countries, are developing breast cancer. Numerous hypotheses have been proposed to explain this dramatic increase in breast cancer incidence in the later stages of life. However, an alternative hypothesis is that aging results in changes in the internal milieu of the organism, such as metabolic, endocrine and immunologic shifts, providing increasingly favorable conditions for tumor induction, promotion and progression. The pineal gland, via its hormone melatonin, has been shown by numerous laboratories to inhibit the proliferation of both human and animal models of breast cancer. As individuals age, there is the onset of disrupted sleep leading to a significant suppression in the nocturnal levels of melatonin after age 60. Using the Buffalo rat as a model, we have begun to characterize the melatonin rhythm in young vs. aged female rats. Our studies as outlined in this report demonstrate that young (2 month old rats) have a robust nocturnal rise in both pineal and serum melatonin levels and that this nocturnal rise is significantly blunted in adult (15 month old) rats. Furthermore, we have demonstrated that in the adult rat, daytime levels of melatonin mtl receptors are significantly depressed and almost undetectable.

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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	9
Reportable Outcomes.....	10
Conclusions.....	10
References.....	10
Appendices.....	10

INTRODUCTION:

An increasing percentage of elderly women, particularly in industrialized countries, are developing breast cancer. Furthermore, it is expected that breast cancer incidence will continue to increase with increased age. A number of hypotheses have been proposed to explain this dramatic increase in breast cancer incidence in the later stages of life, including total dose of carcinogen exposure. However, an alternative hypothesis is that aging results in changes in the internal milieu of the organism, such as metabolic, endocrine and immunologic shifts, providing increasingly favorable conditions for tumor induction, promotion and progression. The pineal gland, via its hormone melatonin, has been shown to play a central role in the regulation of circadian rhythms as well as sleep/wake cycles. Furthermore, the synthesis of melatonin, which is increased in response to darkness, can be blunted and even blocked by exposure to light at night. As individuals age, there is the onset of disrupted sleep and nighttime exposure to light, leads to a significant suppression in the nocturnal levels of melatonin after age 60. Over the last two decades, considerable evidence has accumulated demonstrating that the pineal gland, via its hormone melatonin, possesses significant oncostatic activity, particularly for breast cancer. Our studies have demonstrated that melatonin is able to significantly suppress the transcription of the ER gene, and that it modulates the expression of estrogen-regulated growth-stimulatory factors, oncogenes, and proteins (TGF α , *c-fos*, pS2 and PgR), as well as increasing the expression of the potent growth-inhibitor TGF β , through the activation of a membrane associated G-protein-coupled receptor, the Mella (mtl) receptor. Therefore, given that melatonin levels diminish significantly by the 5th and 6th decades of life as the incidence of breast cancer increases, we hypothesize that *the age related decline in pineal melatonin production leads to an enhancement of breast cancer development and growth in older women*. Given that there are no well designed or tested models of aging and breast cancer the purpose of these studies is to define the age-related changes in melatonin and melatonin receptors, and thus, sensitivity to melatonin in response to age in the female Buffalo rat for the purpose of using the Buffalo rat transplantable mammary tumor model to test the above hypothesis. The scope of the research for this first year was to characterize age related changes in melatonin production and responsivity to melatonin in female Buffalo rats.

BODY:

Our studies have demonstrated that Buffalo rats are quite sensitive to the oncostatic effects of melatonin and to changes in photoperiod. However, the endogenous circadian rhythm of melatonin has not been fully characterized in this rat model. Given that responsiveness to exogenous melatonin is associated with endogenous melatonin synthesis and that the endogenous melatonin rhythm apparently changes with the onset of old age, it is essential that we fully characterize the differences in the circadian melatonin profiles in young, middle aged and old rats. To accomplish these goals we proposed the following Specific Aim.

1. To characterize age related changes in melatonin production and responsivity to melatonin in Buffalo rats by:

- a. Examining melatonin rhythms in young, middle aged, and old Buffalo rats.
- b. To characterizing the expression of the melatonin (mt1) receptor in the hypothalamus and uterus of young, middle aged, and old Buffalo female rats.
- c. Measuring responsivity to melatonin in young, middle aged, and old rats.

To accomplish the studies proposed in this specific aim we proposed following Statement of Work:

First Six Months our tasks, as outlined in the grant were:

- To purchase young, middle aged and old Buffalo rats, and let them adjust to long day photoperiod (12L:12D), then collect serum and measure melatonin serum levels. These studies will define the differences in melatonin levels in young, middle aged and old rats that will serve as the baseline for future studies.

First Year:

- Determine the differences in serum melatonin levels in young, middle aged and old Buffalo rats.
- d.Characterize differences in melatonin receptor (mt1) expression in melatonin-responsive tissues (uteri) in young, middle aged, and old Buffalo rats.

For these studies we purchased female Buffalo rats, BUF (BUF/Ner) (National Cancer Institute) from Charles River Laboratories (Kingston, NY) at 4 weeks of age and maintained in environmentally controlled rooms in facilities (Tulane Vivarium). After 4 weeks in long day photoperiod (12L:12D) two groups of Buffalo rats (10 rats in each group), at 2 month and 15 months of age, corresponding to young, middle aged (adult) rats, respectively, were exsanguinated and truncal blood collected during the light phase (at 0900 and 1600 h), the dark phase (1800, 2000, 2300, 2400, 0100, 0200 and 0400 h) and then again at 0900 h. During the dark phase, blood samples were collected under a dim red light (Kodak Safelight) to avoid light-induced suppression of melatonin production (1). Melatonin levels were measured in the serum over a 24 h period using an ultrasensitive RIA for melatonin (2). We have used this assay previously for the measurement of melatonin levels in Buffalo rats (3). Data from these studies were analyzed by ANOVA simultaneously accounting for sources of variation principally conceived as treatments and time, with repeated measures where indicated.

One problem has developed with this project that we had not anticipated. The supplier of the Buffalo rats, Harlan Sprague-Dawley, no longer maintains aged (20 – 30 month old) rats. Therefore, we have had to purchase these animals and have begun to age them. This has resulted in our examination to date of only young (2 mo) and adult (15 mo) female rats with regards to serum and pineal melatonin levels and mt1 melatonin receptor levels in the uterus. We do anticipate that we will be able to complete the entire project (young, adult and aged rats) close to the proposed deadline.

Serum levels of melatonin: Our data as shown in Figures 1 and 2 demonstrate that in female Buffalo rats nocturnal serum melatonin levels diminish significantly from young rats (8 weeks of age) to adult rats (15 months of age). Figure 1 shows the diurnal rhythm of serum melatonin in young and old rats. As shown in this figure, a significant difference in the timing of the onset or offset of melatonin serum levels is evident between young and adult rats. With lights off at 1800 h (6:00 p.m.) and on at 0600 h (6:00 a.m.) melatonin levels in young rats began to rise between 1800 h and diminish, back to day time values, by 0500 h. In adult rats the onset of melatonin levels during the dark phase of the light:dark cycle was delayed to approximately 2000 h and returned back to daytime values by 0400 h. Thus, adult rats showed at 2-3 h reduction in the plateau of melatonin production. This decrease in the length of the plateau of melatonin was also accompanied by a significant ($p < 0.05$) decrease (29%) in the peak value of serum melatonin as shown in Figure 2. In young rats the mean peak serum levels of melatonin was 123 pg/ml of serum, while in adult rats mean peak serum levels of melatonin was 88 pg/ml.

Figure 1. Changes in female Buffalo rat melatonin rhythm with increasing age. Serum melatonin levels of 10 young (2 months of age) and 10 adult (15 months of age) female Buffalo rats at time points of 0900 and 1600 h (light phase) and 1800, 2000, 2300, 2400, 0100, 0200 and 0400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 before being killed. The curve obtained for both ages is roughly sinusoidal, with low levels during the daytime and a elevated levels at nighttime .

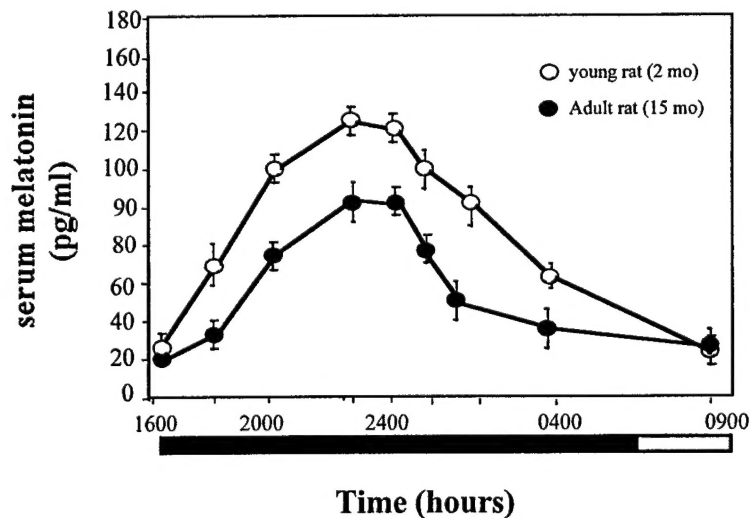
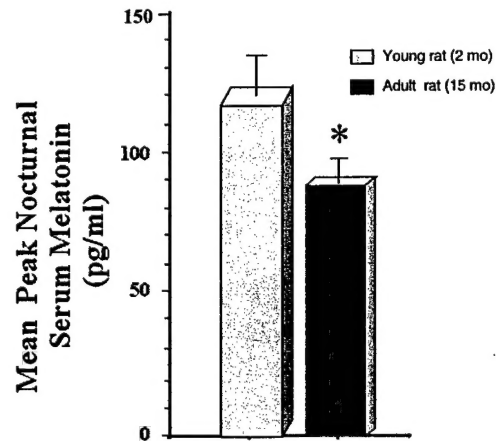
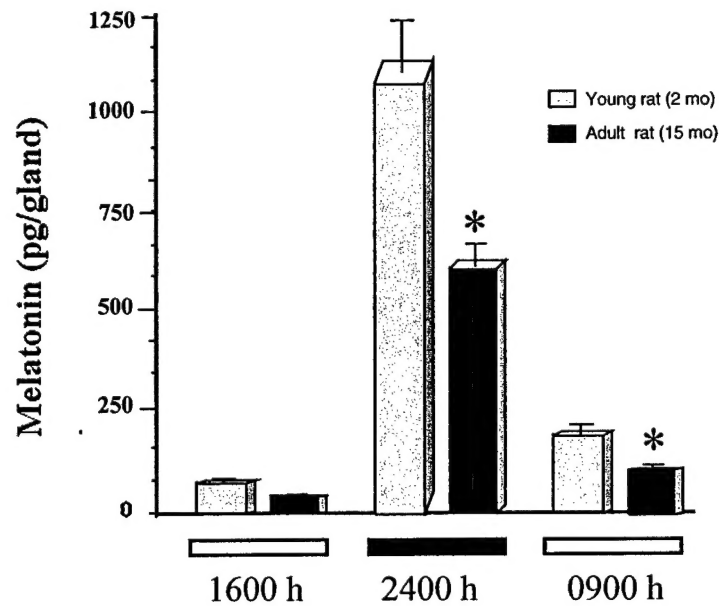


Figure 2. Peak daytime and nighttime serum levels of melatonin in adult and young female Buffalo rats. Serum melatonin levels of 10 young (2 months of age) and 10 adult (15 months of age) female Buffalo rats at 2400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 before being killed under a red-light. * $p < 0.05$ vs. young rats.



Pineal melatonin levels: In addition to examining changes in serum melatonin levels in young and adult Buffalo rats, we also examined pineal levels of melatonin in these same animals. Again, animals were kept in long day photoperiod on a 12 light:12 dark cycle with lights off at 1800 h (6:00 p.m.) and on at 0600 h (6:00 a.m.). After 4 weeks in long day photoperiod (12L:12D) two groups of Buffalo rats (10 rats in each group), at 2 month and 15 months of age, corresponding to young and adult rats, respectively, were exsanguinated and during the light phase (at 0900 h) and the dark phase (2400 h). During the dark phase (2400 h), pineal samples were collected under a dim red light (Kodak Safelight) to avoid light-induced suppression of melatonin, frozen on solid CO₂. Pineal glands were then stored frozen at -20° C until melatonin assays were performed 3-10 days later. Melatonin levels were measured in the serum over a 24 h period using an ultrasensitive RIA for melatonin as described above. Figure 3 shows daytime and nighttime pineal melatonin levels in young (2 months) and middle aged (15 months of age). Middle aged adult rats in this study showed a significant ($p < 0.01$) diminution of nighttime pineal melatonin levels compared to young rats. At this time, the melatonin content of the pineal glands of the young rats exceeded daytime levels by 13-fold, where as in the middle aged, adult, rats only a 7-fold increase in nocturnal levels of pineal melatonin were observed.

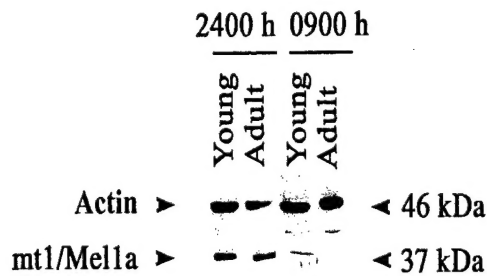
Figure 3. Pineal melatonin content in young and adult female Buffalo rats. Pineal melatonin levels of 7 young (2 months of age) and 10 adult (15 months of age) female Buffalo rats collected at 2400 h (dark phase). Animals were maintained in a light:dark cycle of 12:12 and were exsanguenated under a dark - light. * $p < 0.05$ vs. young rats.



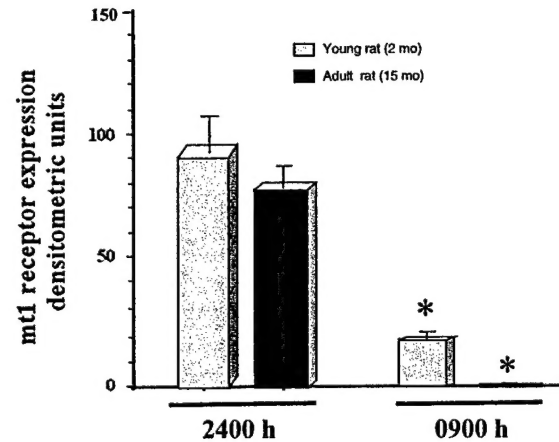
Uterine expression of the melatonin mt1 receptor: It has been well demonstrated that melatonin can modulate uterine function. For example, female hamsters placed in long day photoperiods will have fully developed uteri and administration of melatonin, late in the afternoon, will induce the involution of the uterus. As shown in Figure 4, female Buffalo rats express quantifiable levels of the melatonin mt1 receptor. This figure also shows that uterine levels of the mt1 receptor are diminished by 41% in adult rats compared to young rats.

Figure 4. Uterine melatonin mt1 receptor expression in young and adult female Buffalo rats. (A) Melatonin mt1 receptor protein levels were measured by Western blot analysis, using the 563 anti-mt1 antibody, provided by Dr. Rolf Jockers (Paris, France), from total cellular protein isolated from the uteri of 10 young (2 months of age) and 10 adult (15 months of age) female Buffalo rats at 2400 h (dark phase) and 0900 h (light phase). Animals were maintained in a light:dark cycle of 12:12 and a red-light was used when animals were exsanguinated during the dark phase. (B) Densitometric analysis of mt1 Westerns from 5 different groups of animals. * $p < 0.05$ vs. young rats.

A.



B.



KEY RESEARCH ACCOMPLISHMENTS:

- The onset and offset of the melatonin production was significantly delayed and retracted, respectively, in adult rat as compared to young rats, so that the phase of melatonin production was significantly delayed in adult rats.
- The peak nocturnal serum melatonin level was significantly blunted in adult buffalo, as compared to young rats.
- The nocturnal production of pineal melatonin was significantly blunted (by 2-fold) in the pineal glands of adult (15 month) Buffalo rats as compared to young rats.

REPORTABLE OUTCOMES:

- Army/DoD report
- No other reports have been prepared at this early date

CONCLUSIONS:

The major question addressed in this project is whether melatonin levels and sensitivity are diminished with advancing age and if these changes make aged rats more susceptible to mammary tumor development. In our first phase of this project we have demonstrated that there is a significant 7-fold decline in peak serum melatonin levels in adult female Buffalo rats compared to young rats. In addition, the period of melatonin release is significantly shortened in adult as compared to young rats. These data correlate with the decline in pineal melatonin production in the adult rats as compared to young rats. Finally, we have found that in middle aged rats, that the mt1 receptor is moderately diminished in uterine tissues during the light phase of the light/dark cycle. These studies do not include data from old rats (24-26 months of age) as these rats are still being aged. However, these data demonstrate that there is both a decrease in pineal melatonin production and associated serum melatonin levels as well as diminished levels of the mt1 receptor in adult rats as compared to young rats. Thus, if melatonin does possess antitumor activity with regards to breast cancer, we would expect adult rats to be more susceptible to the formation of mammary tumors, based on their reduced levels of endogenous melatonin. Studies to define the melatonin and mt1 levels in old rats (24-25 months of age) will be conducted in year 2 and followed by studies with transplantable mammary tumors in young, middle age, and old rats in year 3.

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APPENDICES:

None